Plumbagin Content in *Aldrovanda vesiculosa* Shoots

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Introduction

Plumbagin is a yellow naphthoquinone (for its chemical structure see Kovacik & Repcak in another paper of this issue) typically occurring in all genera (*Drosera*, *Dionaea*, *Aldrovanda*) of Droseraceae and widespread within the order Nepenthales (Bonnet *et al.*, 1984; Juniper *et al.*, 1989; Culham & Gornall, 1994, Tokunaga *et al.*, 2004; Schlauer, 2005). Although the presence of plumbagin or its topoisomer, 7-methyljuglone, in Droseraceae organs can very simply be proved by spontaneous sublimation of these substances and staining plastic materials and organic solvents yellow, both the correctness of analytical procedures for quantitative determination of plumbagin and mainly its physiological functions in (carnivorous) plants are still a matter of discussion. Moreover, plumbagin and its derivatives have long been under the interest of pharmacologists for their antimicrobial effects. Tokunaga *et al.* (2004) have recently demonstrated strong cytotoxicity of plumbagin against cancer cells and also its antifeedant effect against insect larvae.

In *Drosera* and *Dionaea* leaves, plumbagin content usually ranges between 2-3% of dry weight (DW; Tokunaga *et al.*, 2004; Kovacik & Repcak, 2006). In the rootless aquatic carnivorous plant *Aldrovanda vesiculosa* L., plumbagin content has never been determined quantitatively. As sun-adapted *Aldrovanda* plants of green temperate European and Asian populations are typically yellow-green and completely lack anthocyanins, a possibly high plumbagin content in this species could be associated with a photoprotective role. The aim of this paper was to investigate plumbagin contents among all available *Aldrovanda* populations, compare two methods of quantitative plumbagin determination in *Aldrovanda*, and to determine plumbagin content in *Aldrovanda* plants of different populations or having been grown at different irradiances. Thus, the possible photoprotective role of plumbagin was tested.

Materials and Methods

Plant material of *Aldrovanda* for plumbagin content determination was collected from both field sites and outdoor or indoor cultures on 20 August 2004. Adult plants originating from E Poland were collected from artificial sites Ptaci blato 1st pool, and Karstejn fen lake in the Trebon region, S Bohemia, Czech Republic (plants were introduced to these sites, see Adamec & Lev, 1999; Adamec, 2005). Plants at Ptaci blato grew in slight shade, while those at Karstejn were collected from three microsites differing greatly among each other in the level of shading by emergent reed vegetation: sun-adapted plants without any shading, shade-adapted plants, and plants growing in very deep shade. The latter plants grew in dense reed stand, evidently at the light minimum threshold for their growth. They were dark green, very short (only 4-5 cm), and weak. Polish plants were also collected from two outdoor plastic cultivation containers in which they had grown in slight shade (Adamec, 1997). Red plants of three Australian tropical populations from outdoor cultivations (N Australia near Darwin, NT; NW Australia from Kimberley,

NT) or an indoor cultivation (N Australia near Katherine, NT) were also collected. They were grown in slight shade (Adamec, 1999). Ripe turions of Polish plants were collected from Ptaci blato and two outdoor containers on 23 October 2004.

For a qualitative test of plumbagin presence, plant material of fourteen *Aldrovanda* populations was collected from small aquaria in an outdoor collection of plants maintained by one of the authors (LA). The original provenances of these plants were: S and N Russia, N Ukraine, Lithuania, E and W Poland, SW Hungary, S Germany (now Switzerland), Japan (near Tokyo), N Australia (Katherine and Darwin), NW, SE, and SW Australia (for most of them see Maldonado San Martin *et al.*, 2003).

Collected plants were thoroughly washed and shoots were divided into two segments, apex with six adult leaf whorls (denoted as "apex") and subsequent six leaf whorls (7th-12th whorls; denoted as "base"). One apical or basal shoot segment (fresh weight 28-145 mg, DW 2.3-12 mg) or one turion (DW ca. 4 mg) was used for one determination. Segments or turions were blotted dry and extracted three times with 1 ml diethyl-ether for about 20 min. before DW of the biomass was estimated. Pooled extracts were alkalized by adding 1 ml 0.1 M NaOH and thoroughly shaken for a few sec. As a result of alkalization, the extract turn orange-red (i.e., bathochromic effect) and plumbagin was dissolved in the aqueous phase. The aqueous phase was diluted ten times with distilled water and optical density measured against blank sample at 400 nm. Plumbagin standard (practical grade P7262, Sigma) was used to obtain calibration curve in the same way. Results are expressed in % DW. Although this method of plumbagin extraction and determination is very simple and fast some objections may arise as to the specificity of this determination. Four parallel plants of each variant were dried shortly at 40°C, wrapped to an aluminum foil, and sent by post to the Laboratory of L. Gastinel (Univ. Limoges, France) for HPLC method of plumbagin determination. After they were overwintered in a refrigerator at 3°C, living turions from Ptaci blato were also sent by post to the Laboratory of L. Gastinel on 17 March.

Here, 3 to 20 mg DW of *Aldrovanda* shoots were extracted in glass tubes with 2 ml of diethyl-ether three times for 30 min. The pooled extracts were allowed to dry overnight by evaporation. The extracts were then resolubilised with 1 ml of pure methanol in the presence of 0.1% formic acid (FA). Twenty-five µl of the samples were injected on an HPLC column (Nucleosil 250 × 4.6 mm 300 Å C18 5µ with a guard column at a 0.5 ml min^{-1} flow rate. Elution of substances absorbing at 400 nm (naphthoquinone ring) was followed by using a 45-min linear gradient from 50% methanol: 50% water with 0.1% FA to 80% methanol: water with 0.1% FA. Quantitative calibration of plumbagin was performed using the plumbagin standard (see above) dissolved in pure methanol with 0.1% FA and injected in the HPLC at different volumes.

MS spectra of the 400 nm absorbing material purified from HPLC gradient (retention time around 21 minutes) were recorded after the injection in the TurboIon ESI spray coupled with Q-trap mass analyser (Applied Biosystems) with parameters, ion spray voltage 5000 V, declustering potential 30 V, flow rate 40 µl min^{-1}. m/z peaks were recorded in the range 50 to 500 amu with the EMS protocol from Analyst 1.4 (Applied Biosystems).

Plumbagin presence was tested using a thin-layer chromatography. One shoot apex of each population, 6-8 mm long, was blotted dry and extracted with 0.25 ml diethyl-ether for 15 min. The extract (20 µl) was dropped on a TLC silica plate F60 and developed first in methanol (to focus the front) and then in toluene for about 15 min. Leaf extract of a non-flowering plant of *Drosera intermedia* was co-chromatographed as standard for plumbagin detection.

Results and Discussion

The presence of plumbagin was clearly determined in all fourteen world populations of *Aldrovanda* but 7-methyljuglone was not detected. Comparing the two methods of plumbagin content determination, direct colorimetric determination (see Table 1) and that using HPLC (see Table 2), there is a much better agreement of the results for apical than for basal segments. Direct colorimetric determination in the same material led to shoot contents greater by 0-90% in apices,

while 2-5 times greater in bases, than using HPLC. There are some possible reasons for this difference. First, the diethyl-ether extract at the direct colorimetric determination might contain also some other ether-extractable substances absorbing at 400 nm, although no such contaminant has been identified so far. Second, due to sublimation of plumbagin, a good deal of this volatile substance could be lost during drying or posting the plant material or solubilized in the plastic phase of the aluminum foil. It is conceivable this loss affected particularly the basal stem segments and Australian plants with more opened leaves or lower biomass, in which the differences between the two methods were more pronounced than in the apices or turions.

Overall, plumbagin content in apical segments ranged between 1.2-4.8% DW and that in basal ones between 0.4-5.0% DW (see Tables 1, 2). The mean plumbagin content of about 2-3% DW in *Aldrovanda* apical shoot segments corresponds to that found in two *Drosera* species (1.9-3.0% DW; Kovacik & Repcak, 2006) or in *Dionaea muscipula* (about 3 % DW; Tokunaga *et al.* 2004). Thus, plumbagin content in *Aldrovanda* shoot is not greater than in other genera of Droseraceae. However, with either assay method, plumbagin content was evidently greater in apical than basal shoot segments in Polish plants but this difference was not too distinct in dark-red Australian plants (see Tables 1, 2). This gradient in plumbagin content in *Aldrovanda* shoot segments of different age is in a good agreement with Repcak *et al.* (2000) who found a decrease of 7-methyljuglone content in older leaves of *Drosera* spp. Obviously, plumbagin is released from old and ageing tissues of *Aldrovanda* to ambient medium (Adamec, unpubl.). In *Aldrovanda*, plumbagin content in shoot apices was similar to that in turions (Tables 1, 2). The data on plumbagin content do not support the hypothesis that this substance has a specific and light-regulated photoprotective role in *Aldrovanda* as the content was the same in both sun-and shade-adapted plants. Nevertheless, due to optical properties of plumbagin (absorption of blue light and UV), it is possible to assume certain role in photoprotection. These results support the view that the main and primary role of plumbagin in Droseraceae is antifeedant and antimicrobial, thus protecting the plants against herbivores and microbial parasites (Tokunaga *et al.* 2004).

Origin and light exposure of plant material	Plant A		Plant B	
	Apex	Base	Apex	Base
Shoot segments				
E Poland: Ptaci blato 1st pool, slight shade	3.34	1.66	3.13	1.67
E Poland: Karstejn fen lake, sun-adapted	2.75	2.00	2.54	1.68
E Poland: Karstejn fen lake, shade-adapted	2.18	1.70	2.37	1.47
E Poland: Karstejn fen lake, very deep shade	2.31	1.46	3.04	2.49
E Poland: culture 1; small container, slight shade	3.78	2.78	3.64	2.54
N Australia, Katherine: indoor aquarium, slight shade	4.55	3.41	5.95	3.93
Turions	Turion A		Turion B	
E Poland: Ptaci blato 1st pool, slight shade	2.68		3.21	
E Poland: culture 1; small container, shade	2.69		2.39	
E Poland: culture II; big container, shade	3.50		3.68	

Table 1: Content of plumbagin (in % of DW) in apical and basal shoot segments of different strains and in ripe turions of *Aldrovanda vesiculosa*. Apical shoot segments contained shoot apex + the first six adult leaf whorls, while the basal ones the subsequent six leaf whorls (i.e., the 7th-12th ones). Results for two different plants or turions A and B are based on measurement of optical density at 400 nm of alkalized diethyl-ether extract.

Origin and light exposure of plant material	Plumb. content (% DW)	
	Apex	Base
Ptaci blato	2.2±0.9[a]	0.4±0.07[b]
Karstejn: sun-adapted	2.1±0.9[a]	0.5±0.05[b]
Karstejn: shade-adapted	2.4±0.4[a]	0.7±0.1[b]
Karstejn: deep shade-adapted	1.2±0.4	—
Culture I: small container	2.0±0.7[a]	0.5±0.1[b]
NW Australian: outdoors	1.2±0.2[a]	0.5±0.06[b]
Turions-Ptaci blato: 1st pool	2.4+0.5	

Table 2: Plumbagin content determined in diethyl-ether-extracted dry *Aldrovanda* biomass using HPLC. For apical and basal segments, see Table 1. Except for Australian plants, the others were from E Poland. Means+SD of 3-4 independent analyses are shown. The different letters within the same raw denote statistically significant difference at $P<0.05$ (t-test).

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Looking Back: CPN 25 years ago

Previous CPN coeditor and ICPS cofounder Don Schnell published a now-classic review of all the infraspecific *Sarracenia purpurea* taxa then known, including a variation that resulted because of its growing in marl fens. After twenty-five years, this treatment would be modified by the addition of *Sarracenia purpurea* subsp. *venosa* var. *montana*, *Sarracenia purpurea* subsp. *venosa* var. *burkei* f. *luteola*, perhaps a nod to the veinless expression of *Sarracenia purpurea* subsp. *purpurea*. Of course a modern author would also have to address the issue of whether to accept the *Sarracenia rosea* species as having merit.